ISSN 2581-6217

World Journal of Pharmaceutical Science & Technology

Journal homepage: www.wjpst.com

Original Research Article

CONCISE REVIEW ON METHODS FOR DETERMINATION OF DIURETIC ACTIVITY.

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Received: 15-03-2021, Revised: 25-04-2021, Accepted: 28-04-2021

ABSTRACT

There is increasing interest in the health and wellness benefits of herbs and botanicals. Natural medicine is a precious resource of therapeutically active components compounds and has increasingly attracted the attention of researchers; further many studies have reported that herbal diuretics might be a useful tool in the treatment of hypertension and chronic kidney diseases. There are a growing number of studies purporting diuretic effects with traditional medicines.

Diuretics drugs increase the rate of urine flow and adjust the volume and composition of body fluids. Drug-induced diuresis is beneficial for the treatment of many maladies such as congestive heart failure (CHF), chronic renal failure, nephritis, cirrhosis, hypertension and pregnancy-induced toxemia. Medicinal herbs are the significant source as Diuretics. Mono and poly-herbal preparations have been used as diuretics. According to one estimate, more than 650 mono and poly-herbal preparations in the form of decoction, tincture, tablets and capsules from more than 75 plants are in clinical use. The various methods for screening of diuretic agents provides useful tool to evaluate the safety and effectiveness of the drugs. It is also useful for determining the dose level of particular class of diuretic agents. (1 -5)

The present article gives a brief review about different methods *(in-vitro and in-vivo)* used for studying diuretic activity.

KEYWORDS: Diuretic activity, herbs and botanicals, furosemide, albino mice, dogs, saluresis,

INTRODUCTION:

High blood pressure represents an important risk factor to development of other cardiovascular diseases and constitutes one of the main causes of mortality in the world. (6) Diuretic compounds that stimulate the excretion of water are potentially useful in most of disorders including those exhibiting oedema such as congestive heart failure, nephritis, toxemia of pregnancy, premenstrual tension and hypertension.(7) Diuretic can also increase the elimination of electrolytes.(8) The modern era of diuretic therapy began in 1949 when sulphanilamide was discovered to possess diuretic and natriuretic properties.(9) The net excretory effect of diuretic agents causes changes in urine flow, pH, and ionic compositions of urine and blood.(10)

However, many of the diuretics currently used in clinical practice have been associated with a number of adverse effects, including electrolyte imbalance, metabolic alterations, the onset of diabetes, activation of the renin- angiotensin and neuroendocrine systems, and impairment of sexual function. Therefore, it is important to consider alternatives that have greater effectiveness and fewer side effects. Many of the herbs used in folk medicine have yet to be scientifically evaluated for their effectiveness and safety. (3)

For thousands of years, humans have been using diuretics to reduce the water retention caused by some health conditions such as high blood pressure, heart diseases or pre-menstrual syndrome, among others. Although there is a wide therapeutic stock of synthetic drugs that belong to this pharmacological group, a considerable amount of decoctions and in fusions of medicinal plants are used to reduce fluid retention. But the diuretic effectiveness of this kind of medicinal plants needs to be experimentally proved, because diuresis could be influenced not only by the form of administration (infusion or decoction) which implies the consumption of a great amount of liquids that can provoke an increase in the volume of urine excreted without a true evidence of a diuretic action, but also by the difficulty of obtaining reproducible data involving a larger number of animals.(11)

Table 1: Classification and adverse effects of diuretics (12,13)

Class of diuretic	Examples	Adverse Effects
Thiazides and related diuretics	Bendroflumethiazide Chlorthalidone Cyclopenthiazide Indapamide Metolazone Xipamide	Orthostatic hypotension. Decreased serum Na^+, K^+, Mg^+, and H^+. Modest increases in Ca^{2+}. Increases in serum uric acid, glucose, cholesterol, LDL, and triglycerides.

		Erectile dysfunction, impotence, and lithium accumulation.
Carbonic anhydrase inhibitors	Acetazolamide	Volume depletion, hypokalemia, hyperchloremic metabolic acidosis, light-headedness, circumoral paresthesias, weakness, and confusion
Loop diuretics	Furosemide Bumetanide Torasemide	Volume depletion, decreased serum K^+, Na^+, Mg^+, and H^+. Increased uric acid, glucose, cholesterol, LDL, and triglycerides. Nausea, ototoxicity, and allergic interstitial nephritis.
Osmotic diuretics	Mannitol	Low volume, K^+, and H^+. CHF, headache, nausea, vomit, fever, confusion, and lethargic state.
Potassium-sparing diuretics	Amiloride Triamterine	Increase serum K^+, Cl^-, & H^+. Nausea, flatulence & skin rash with amiloride or triamterene, nephrolithiasis with triamterene.
Potassium-sparing diuretics and aldosterone antagonists	Spironolactone Eplerenone	Gynecomastia & decreased libido in men with spironolactone

Plants evaluated for diuretic activity:

Human beings utilize many species of flora for food and medicine. It is also expected that the traditional and modern medicine uses about 50,000 - 70,000 species of plants. (8) Herbal drugs have gained importance and popularity in recent years because of their safety, efficacy and cost effectiveness.(4) Medicinal plants can be significant sources of undiscovered chemical substances with potential therapeutic effects. In fact, the World Health Organization has estimated that over 75% of the world's population still relies on plant-derived medicines, usually obtained from traditional healers, for basic healthcare needs.(14) Regardless of their specific pharmacological effects, most medicinal plants have been described as having a more or less pronounced diuretic effect. However, very few studies have examined the mechanisms of action of inducing renal excretion.(6) Some of the diuretics are derived from medicinal plants and a vast number of medicinal plants mentioned in ayurvedic system of medicine are known to possess diuretic properties such as Abelmoschus esculentus, Bacopa monnieri, Barbara vulgaris and Cissampelos pareira.(7)

Number of species and genuses reporting diuretic effects. of these, the most promising, at the present time, are the species *Foeniculum vulgare, Fraxinus excelsior, Hibiscus sabdariffa, Petroselinum sativum and Spergularia purpurea, and species from the genuses Cucumis (Cucumis melo and Cucumis trigonus), Equisetum (Equisetum bogotense, Equisetum fluviatile, Equisetum giganteum, Equisetum hiemale var. affine and Equisetum myriochaetum), Lepidium (Lepidium latifolium and Lepidium sativum), Phyllanthus (Phyllanthus amarus,Phyllanthus corcovadensis and Phyllanthus sellowianus) and Sambucus (Sambucus*

mexicana and Sambucus nigra). Many indigenous drugs have been claimed to have diuretic effect in Ayurvedic system. Among the several plants, Crataeva nurvala, Dolichos biflorus, Tribulus terrestris, Dendrophthoe falcata, Boerhaavia diffusa, Saccharum officinarum, Butea frondosa, Boerhaavia repens, Boerhaavia rependa, Homonia riparia, Centratherum anthelmintivum, Vitis venifera and Duranta repens have shown excellent diuretic activity.(15)

METHODS TO DETERMINE DIURETIC ACTIVITY:

A) In-Vitro Methods

1) Carbonic Anhydrase Inhibition *In-Vitro* (16,17)

Carbonic anhydrase inhibitors were originally used as diuretics but are no longer used for this purpose except in limited circumstances (metabolic alkalosis accompanied by edema). Numerous isoenzymes exist, of which CA II is the most abundant in proximal tubules and erythrocytes. The activity of carbonic anhydrase in erythrocytes is significantly higher than that in the kidney, and the enzyme is easily harvested by lysing mammalian whole blood. Thus, examination of the degree of inhibition of erythrocyte carbonic anhydrase may serve as a suitable surrogate for examination of effects on renal activity. In spite of the fact that newer diuretics are based on other modes of action, the test for inhibition of carbonic anhydrase should be performed for evaluation of a new compound.

The analytical method is based on the catalysis of the conversion of CO_2 to H_2CO_3 by the enzyme, with resulting decrease in pH being monitored colorimetrically.

Requirement:

- Phenol red indicator solution: 12.5 mg phenol red/l 2.6 mM $NaHCO_3$, pH 8.3 + 218 mM Na_2CO_3
- 1 M sodium carbonate/bicarbonate buffer, pH 9.8
- Enzyme: carbonic anhydrase from dog blood; blood is collected into a heparinized tube and diluted 1:100 with deionized water.
- Equipment
    - Reaction vessel
    - Monostat bench-mounted flowmeter
    - 30 % CO_2.

Procedure:

The analytical method is based on the catalysis of the conversion of CO_2 to H_2CO_3 by the enzyme, with resulting decrease in pH being monitored colorimetrically. CO_2 flow rate is adjusted to 30 ml/min. The following solutions are added to the reaction vessel:

- 400 µl phenol red indicator solution
- 100 µl enzyme
- 200 µl H2O or appropriate drug concentration after 3 min for equilibration
- 100 µl carbonate/bicarbonate buffer is added.

Evaluation/Expression of results:

The following parameters are determined in duplicate samples:

T_u (uncatalyzed time) = time for the color change to occur in the absence of enzyme

T_e (catalyzed time) = time for the color change to occur in the presence of the enzyme

$T_u - Te$ = enzyme rate

T_i = enzyme rate in the presence of various concentrations of inhibitor.

Percent inhibition of carbonic anhydrase is calculated according to the following formula:

$$\%\,\text{Inhibition} = 1 - \frac{(T_u - T_e) - (T_i - T_e)}{T_u - T_e} \times 100$$

Determination of carbonic anhydrase inhibition is of value to characterize the activity spectrum of sulfonamide diuretics.

2) Patch Clamp Technique in Kidney Cells (16, 18)

In the different parts of the kidney (proximal tubules, distal tubules, collecting ducts) fluid is reabsorbed and substances may be transported either from the tubule lumen to the blood side (reabsorption) or vice versa (secretion). The various modes of the patch clamp technique (cell-attached, cell-excised, whole-cell mode). The impact of this technique has been far-reaching and has been applied to a wide range of cell types, with particular relevance to excitable cells such as neurons and cardiomyocytes.

Procedure:

Segments of late superficial proximal tubules of rabbit kidney are dissected and perfused from one end with a perfusion system. The non-cannulated end of the tubule is freely accessible to a patch pipette. Under optical control (differential interference contrast optics with 400×magnification) the patch pipette can be moved through the open end into the tubule lumen and is brought in contact with the brush border membrane. After slight suction of the patch electrode, gigaseals form instantaneously and single potassium or sodium channels can be recorded in the cell-attached or inside-out cell excised mode. In order to obtain exposed lateral cell membranes suitable to the application of the patch clamp method, pieces of the tubule are torn off by means of a glass pipette (diameter about 40 μm). As to facilitate the tearing off, the tubules are incubated for about 5min in 0.5 g/l collagenase at room temperature. After tearing off part of the cannulated tubule, clean lateral cell membranes are exposed at the non-cannulated end. The patch pipette can be moved to the lateral cell membrane and gigaseals can be obtained.

It was possible, to investigate potassium channels and nonselective cation in these membranes.

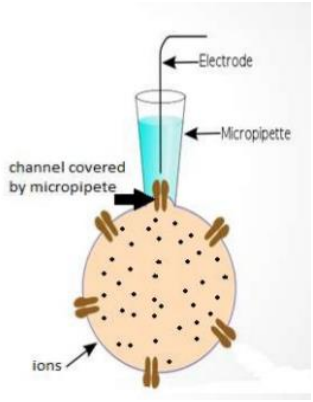

Fig 1: Patch clamp technique

Evaluation/Expression of results:

In isolated perfused renal tubules, concentration response curves of drugs which inhibit ion channels can be obtained with the patch clamp technique.

In isolated cells of the proximal tubule, the whole-cell mode of the patch clamp technique enables the investigation of the sodium-alanine cotransport system. The apparent *K*m values for sodium and L-alanine can be recorded.

Limitations:

As cells are still part of an epithelial layer and, therefore, are intracellularly coupled, the whole-cell technique is not appropriate in this preparation. Additionally need for high-quality cell suspensions and the 'blind' nature of the cell selection process for patching.

3) Perfusion of Isolated Kidney Tubules (16)

The *in vitro* perfusion of isolated tubule segments is the method of choice if one has to identify the site and the mechanism of action of a pharmacological agent which has been shown to act on kidney function in clearance and micropuncture studies. This translates into limitations with non-uniform cell suspensions**.**

Procedure:

Kidney tubule segments of several species: man; rabbit; rat; mouse; hamster; snake; birds etc. The tubule segments are dissected from thin kidney slices (< 1 mm thickness). Usually dissection can be done using sharpened forceps or needles without the addition of proteases (collagenase).

A 20–50× lens is used for dissection. Dark field illumination is helpful for the identification of the segment under study.

The dissection of DTL, ATL and PCD is much more difficult because these segments are damaged easily by the mechanical dissection. Dissection is usually performed at 4°C in a Ringer type solution.

The dissected segment is transferred into the perfusion chamber by a transfer pipette. The perfusion chamber is mounted in the stage of an inverted microscope (20–400×). The chamber is usually kept at 37°C, and the bath perfusate is also preheated to this temperature.

The bath perfusate will depend on the tubule segment under study. In most instances it will contain HCO_3^- and will be bubbled with CO_2.

The metabolic substrate will be acetate for PT and D-glucose for TAL, CCD etc. The actual perfusion is performed with two sets of concentric glass pipettes one set at the perfusion end; and one at the collection end of the segment.

The glass tube is rotated at approximately 1 rps, is moved in perpendicular direction by a remote control, and the heating filament is moved in xy direction also by a remote control. The shaping of the glass is observed continuously by a lens (5–50×).

4) Isolated Perfused Kidney (16)

Isolated kidney is a good tool for studying proximal tubule, but of limited value for distal tubule function. The kidney can be perfused *in situ* and isolated *in vitro*. The isolated kidney can be perfused by a pump using blood or plasma-like solutions.

Procedure:

Kidneys are obtained from anaesthetized male rats with a body weight of 300 to 400 g. The donor animals are fasted overnight prior to surgery, but have free access to water. After the abdominal cavity is exposed by a ventricular incision, the right ureter is cannulated with PE-50 polyethylene tubing and heparin is injected into the vena cava (500 U/kg body weight).

The venous cannula is introduced into the vena cava below the right renal vein. The right kidney is freed from the perirenal fat, not disrupting the renal capsule.

The renal artery is cannulated via the superior mesenteric artery without interruption of flow. Thereafter, the kidney is continuously perfused with a perfusion solution fed from the gravity system situated 130cm above the cannula. Ligatures around the renal artery and vena cava above the renal pedicle are tied. The kidney is then removed from the animal and placed in a Plexiglas chamber. A perfusion pressure of 80–90mmHg in the renal artery is maintained by adjusting the speed of the perfusion pump. For moredetails see references.

Evaluation:

After the equilibration period, clearance periods of 20 min are used. Urine samples are collected and perfusate is obtained at midpoint of the clearance period for the evaluation of overall kidney function. For determination of glomerular filtration rate (GFR) and fluid transport, 3H-labelled polyethylene glycol is added to a modified Krebs-Henseleit bicarbonate buffer. Electrolytes are determined in urine by standard flame photometry.

Fractional excretions of water, electrolytes and test compounds are calculated.

Limitation:

One specific problem of the blood-perfused dog kidney *in vitro* is its instability. After only 1 h of perfusion, glomerular filtration and renal blood flow decline markedly.

Isolated perfused dog kidneys have been reported to be less stable than those of other species, with glomerular filtration and renal flow markedly decreasing after only one hour of perfusion. The *in situ*-perfused isolated

dog kidney seems to be more stable. Distal tubule functions are impaired in isolated perfused kidneys in all species. Urine acidification, concentration and dilution functions are also abnormal, and effects on these cannot be assessed in these models.

B) *In-Vivo* Methods

1) Diuretic Activity in Rats (LIPSCHITZ Test) (16.19,20,21)

The Lipschitz test has been proven to be a standard method and a very useful tool for screening of potential diuretics. A method for testing diuretic activity in rats has been described by Lipschitz et al. (1943). The test is based on water and sodium excretion in test animals and compared to rats treated with a high dose of urea. A method using rats for estimation of antidiuretic potency was described by Burn in 1931. This method or a modification of it has been used for diuretic assays by most of the subsequent workers. In 1943, Lipchitz et at. described a method suitable for diuretic assay using several commonly used diuretics. This method by itself is a modification of Burn's method. Since 1943, most of the workers have used the method of Lipschitz with some Modifications.

Requirement:

- Male Wistar rats weighing 100–200 g
- Metabolic cage with a wire mesh bottom
- Minimum Three animals per group
- rats are fed with standard diet (Altromin pellets) and water ad libitum
- Fifteen hours prior to the experiment food and water are withdrawn
- Urine excretion is recorded after 5 and after 24 h.

Fig 2: Metabolic cage

Procedure:

Immediately after administration, the mice were placed in metabolism cages. All the doses were administered intragastrically by gastric canula. (15)

- Albino rats of either sex weighing 100-200 g were selected and are fasted overnight and saline is administered orally.
- These rats to be divided in four groups as:
  - Group I : Only normal saline (Negative control)
  - Group II: Saline + Furosemide (Positive control)
  - Group III: Saline + Pulp extract
  - Group IV: Saline + Seed extract
- Immediately after the administration the animals to be kept in metabolic cages (3 /cage) specially designed to separate urine and fecal matter and kept at room temperature of 25 ± 0.5º C
- The total volume of urine to be collected at the end of 5 hrs after dosing. During this period no water and food was made available to animals.

Na+ and K+ concentrations can be measured using flame photometer.

Evaluation/Expression of results:

Results are expressed as the "Lipschitz-value" *i. e.* the ratio T/U,

in which T is the response of the test compound, and U, that of urea treatment.

Indices of 1.0 and more are regarded as a positive effect. With potent diuretics, Lipschitz values of 2.0 and more can be found.

Calculating this index for the 24 h excretion period as well as for 5 h indicates the duration of the diuretic effect.

$$\text{Diuretic index} = \frac{Urinary\ excretion\ of\ treated\ group}{Urinary\ excretion\ of control\ group}$$

$$\text{Diuretic activity} = \frac{Diuretic\ action\ of\ test\ drug}{Diuretic\ action\ of\ standard\ drug}$$

$$\text{Saliuretic index} = \frac{Concentration\ of\ electrolytes\ in\ urine\ of\ test\ group}{Concentration\ of\ electrolytes\ in\ urine\ of\ control}$$

Table 2: Urine volume, urinary electrolytes and sailuretic index of different groups

	Urine volume (ml/kg)	**Na^+ mmol/ L**	**K^+ mmol/ L**	**Cl^- mmol/ L**	**Sailuretic index**	
					Na^+	**K^+**
Negative control (Group I)						
Positive control (Group I)						
Group III						

Group IV						

Advantages:

Rat, can and has been used for accurate bioassays of new diuretics. Outstanding advantages of rat method are, it is economical, simple, reliable, more consistent and only small amounts of drugs are sufficient. Rats gave much more consistent results than dogs.

2) Saluretic Activity in Rats (16, 17, 22, 23)

Excretion of electrolytes is as important as the excretion of water for treatment of peripheral edema and ascites in congestive heart failure as well as for treatment of hypertension. Potassium loss has to be avoided. As a consequence, saluretic drugs and potassium-sparing diuretics were developed. The diuresis test in rats was modified in such a way that potassium and chloride as well as osmolality are determined in addition to water and sodium. Ratios between electrolytes can be calculated indicating carbonic anhydrase inhibition or a potassium sparing effect.

Requirement:

- Male Wistar rats weighing 100–200 g
- Metabolic cage with a wire mesh bottom
- Minimum Three animals per group
- Rats are fed with standard diet (Altromin pellets) and water ad libitum
- Fifteen hours prior to the experiment food and water are withdrawn
- Urine excretion is recorded after 5 and after 24 h.
- Furosemide (25 mg/kg p.o.), hydrochlorothiazide (25mg/kg p.o.), triamterene (50 mg/kg p.o.), or amiloride (50mg/kg p.o.) can used as standards.

Procedure:

Male Wistar rats weighing 100–200 g fed with standard diet (Altromin pellets) and water ad libitum are used. Fifteen hours prior to the test, food but not water is withdrawn. Test compounds are applied in a dose of 50 mg/kg orally in 0.5ml/100 g body weight starch suspension. Three animals are placed in one metabolic cage provided with a wire mesh bottom and a funnel to collect the urine. Two groups of 3 animals are used for each dose of a test drug.

Urine excretion is registered every hour up to 5 h. Samples can be stored at − 20°C for further analyses. The rats were maintained at 21 ± 2 ºC, relative humidity 40 % − 60 %.

Evaluation/Expression of results:

- The sum of Na^+ and Cl^- excretion is calculated as parameter for saluretic activity.
- The ratio Na^+/K^+ is calculated for natriuretic activity. Values greater than 2.0 indicate a favorable natriuretic effect. Ratios greater than 10.0 indicate a potassium-sparing effect.
- The ratio $Cl^-/Na^+ + K^+$ (ion quotient) is calculated to estimate carbonic anhydrase inhibition.

- Carbonic anhydrase inhibition can be excluded at ratios between 1.0 and 0.8. With decreasing ratios slight to strong carbonic anhydrase inhibition can be assumed.

3) Diuretic and Saluretic Activity in Dogs (16, 17)

Dogs have been extensively used to study renal physiology and the action of diuretics. Renal physiology of the dog is claimed to be closer to man than that of rats. Oral absorbability of diuretic substances can appropriately be studied in dogs. Using catheters, interval collections of urine can be made with more reliability than in rats. Simultaneously, blood samples can be withdrawn to study pharmacokinetics.

Procedure:

- Beagle dogs of either sex have to undergo intensive training to be accustomed to accept gavage feeding and hourly catheterization without any resistance.
- The dogs are placed in metabolic cages. At least four dogs are used as controls receiving water only, as standard controls (1 g/kg urea p.o. or 5 mg/kg furosemide p.o.), or the test drug group.
- Twenty-four hours prior to the experiment, food but not water is withheld. On the morning of the experiment, the urine bladder is emptied with a plastic catheter.
- The dogs receive 20 ml/kg body weight water by gavage, followed by hourly doses of 4 ml/kg body weight drinking water. The bladder is catheterized twice in an interval of 1 h and the urine collected for analysis of initial values.
- Then, the test compound or the standard is applied either orally or intravenously. Hourly catheterization is repeated over the next 6 h. Without further water dosage, the animals are placed in metabolic cages overnight. Twenty-four hours after dosage of the test compound, the dogs are catheterized once more and this urine together with the urine collected overnight in the metabolic cage registered.
- All urine samples are analyzed by flame photometry for sodium and potassium and by argentometry (Chloride Titrator Aminco) for chloride content. Furthermore, osmolality is measured with an osmometer.

Evaluation:

Urine volume, electrolyte concentrations, and osmolality are averaged for each group. The values are plotted against time to allow comparison with pretreatment values as well as with water controls and standards. The nonparametric U-test is used for statistical analysis.

4) Clearance Methods (16)

Procedure:

Clearance experiments are performed either in conscious or anaesthetized beagle dogs under conditions of water diuresis and hydropenia Water diuresis is induced by oral administration of 50ml of water per kg body weight andmaintained by continuos infusion into jugular vein of 2.5 % glucose solution and 0.58 % NaCl solution at 0.5 ml/min per kg body weight. When water diuresis is well established, the glucose infusion is discontinued and control urine samples are collected by urethral catheter. Blood samples are obtained in the

middle of each clearance period. After the control period, compounds to be tested are administered and further clearance tests are performed.

Requirement:

Hydropenia is induced by withdrawing the drinking water 48 h before experiment. On the day before the experiment 0.5 U/kg body weight of vasopressin in oil is injected intramuscularly.

On the day of the experiment 20mU/kg vasopressin is injected i.v., followed by infusion of 50 mU/kg per hour vasopressin. To accomplish constant urine flow 5 % NaCl solution is infused at 1 ml/min per kg body weight up to i.v. administration of a compound to be tested, followed by i.v. infusion of 0.9 % NaCl solution at a rate equal to the

urine flow.

Evaluation:

Glomerular filtration rate (GFR) and renal plasma flow (RPF) are measured by the clearance of inulin and para-aminohippurate, respectively. Inulin and paraaminohippurate are measured Tracers are administered intravenously to achieve near steady-state concentrations; a priming dose loads the plasma and extracellular compartments, and subsequent infusion replaces renal losses. Once steady state plasma tracer levels are approached, a series of timed urine collections (clearance periods) are performed.

Tracers for the determination of GFR must be freely filtered and then neither secreted nor reabsorbed. This allows the assumption that the amount of plasma cleared of the tracer per unit time represents that which has been filtered through the glomeruli (*i.e.* GFR). The fructose polysaccharide, inulin (mw ~5200) is the most commonly used tracer in all species and serves as the 'gold standard' to which others are compared.

Inulin is measured colorimetrically, either by acid hydrolysis to generate a green product, or by a series of enzymatic reactions based on inulinase with subsequent reduction of NADH. HPLC methods are used for the remaining exogenous GFR tracers. PAH and TEA are measured colorimetrically.

Renal clearance (Cl) of any compound (X) can be determined by comparing the urinary excretion rate of compound X to the plasma concentration of compound X. The urinary excretion rate is calculated as:

$$\text{Urinary excretion rate (mg/min)} = U_x\,(\text{mg/ml}) \times V(\text{ml/min})$$

Where Ux represents the concentration of substance X in urine (in mg/ml) and V represents the volume of urine collected per unit time (in ml/min). Thus, the clearance equation may be constructed:

$$Cl_x(\text{ml/minute}) = U_x\,(\text{mg/ml}) \times V(\text{ml/min})/P_x\,(\text{mg/ml})$$

Where Px is the concentration of compound X in plasma (in mg/ml)

GFR is estimated by calculating the clearance of the tracer or endogenous substance. RPF estimated using PAH clearance is often designated effective renal plasma flow (ERPF). Renal plasma flow is converted to

renal blood flow (RBF) by dividing ERPF by the plasma fraction of whole blood, as estimated from the hematocrit (Hct):

$$RBF = ERPF/(1 - Hct)$$

The clearances of other compounds can be compared with inulin clearances to determine how the kidney functions in the elimination of the test compound.

A clearance ratio is constructed by dividing the renal clearance of the test compound (X) by the renal clearance of inulin:

$$\text{Clearance Ratio} = CL_x\ ,\ (\text{ml/min})/C_{\text{inulin}},\ (\text{ml/min})$$

A clearance ratio < 1.0 indicates reabsorption of the test substance following filtration, whereas active secretion will result in a clearance ratio of > 1.0.

Limitation: First-pass extraction of PAH is highly variable both between species and between individuals within a species, which adds to the inherent inaccuracy of the estimate of RBF by this method. Furthermore, the test compound may interfere with the extraction of either PAH or TEA by competing for transport by the organic anion or cation transporters. Exogenous creatinine clearance compensates for the insensitivity of the method as well as the interference by endogenous chromagens by artificially increasing the plasma creatinine concentration.

5) Micropuncture Techniques in the Rat (16)

Micropuncture techniques have been applied to the direct investigation of the effect of diuretics on single nephron function. The observed changes in tubular fluid reabsorptive rates and electrolyte concentrations can be used to asses the mechanismof action. The rat is the model of choice since proximal and distal tubules as well as collecting ducts are accessible for micropuncture.

Procedure:

Clearance and free-flow micropuncture studies are performed in rats with a body weight of about 250 g, anaesthetized by the intraperitoneal injection of thiopentone (Trapanal 50 mg/kg).

The animals are fasted for 16 h before the beginning of the experiment, but have free access to tap water. After anesthesia the animals are placed on a thermostatically heated table. Thereafter rats are tracheotomized and carotid artery and jugular vein are cannulated for blood pressure recording, blood sampling, and for infusion of compounds, respectively.

The left kidney is carefully exposed by a flank incision, embedded in a small plastic vessel with cotton wool, and bathed with paraffin oil at 37°C. The ureter is cannulated and rectal temperature monitored continuously. A bolus injection of 75 μCi inulin 3H in 0.7ml NaCl solution is given, followed by 0.85 % NaCl solution at a rate of 2.5 ml/min per 100 g body weight. The sustained infusion delivers 75 μCi inulin 3H per hour. The control puncture of tubules is performed 45 min after beginning of the intravenous infusion.

The direct collection of tubular fluid samples from proximal and distal tubules is carried out with glass capillaries of 8 to 10 μm external diameter using a micromanipulator and microscopic observation. Distal tubules are identified by intravenous injection of lissamine green.

The control period is followed by the test period. After an equilibration period of 30min with the compound to be tested, micropuncture is performed again and tubular fluid is collected.

The uretral urine is collected and blood sampling is performed in the middle of each clearance period. The infusion rate/volume is determined by the size of the animal. Distal collection is done by identification of the perfused segment by its dye content; a second micropipette is then inserted, a distal oil or wax block is placed and the infusion fluid can be collected for subsequent analysis.

Evaluation:

The following parameters may be determined: inulin clearance (GFR), single nephron GFR, fractional delivery of water, sodium and potassium in proximal and distal tubules and in urine. Fluid reabsorption (Jv) is assessed by comparing the inulin concentration in the perfusate to that in the collected sample, thus

$$Jv = V0 - VL,$$

Where VL is the collected fluid volume (normalized for the length of the tubular segment over which it was collected)

$$V0 = VL(INL/IN0),$$

Where INL= concentration of inulin in the collected sample, and IN0 is the inulin concentration in the perfusate.

The net flux of the desired analyte (JX, where X is the anlyte of interest – Na, K, HCO3, glucose, etc.), is calculated thus:

$$JX = V0[X]0 - VL[X]L,$$

Where [X]0 is the concentration of the analyte in the perfusion fluid, and [X]L is the concentration of the analyte in the collected fluid.

ACKNOWLEDGEMENT:

The authors are grateful to the authorities of Department of Pharmacology, Dr. V. Vikhe Patil College of Pharmacy, Vilad Ghat, Dist-Ahmednagar, M.H., India for providing facilities to carry our review.

CONFLICT OF INTEREST:

The authors declare no conflict of interest.

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